(54) Title: METHODS AND COMPOSITIONS FOR GENETIC THERAPY AND POTENTIATION OF ANTI-TUMOR IMMUNITY
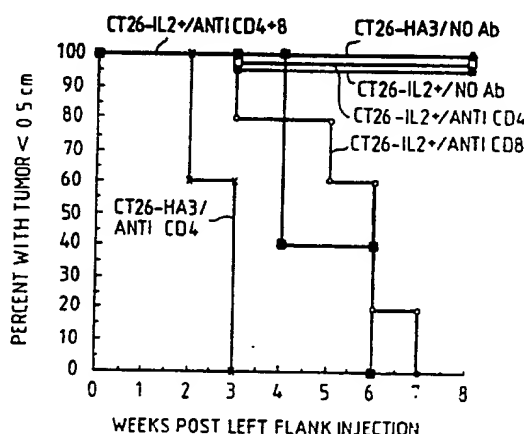
(57) Abstract

The invention provides a preparation of tumor cell lines containing an exogenous gene encoding a polypeptide that potentiates the immune response to the tumor, for example, interleukin-2. The invention includes methods for cancer therapy by immunization with the immunopotentiating tumor cell. The invention also provides methodology whereby one can selectively ablate in vitro and in vivo genetically altered cells. A preferred embodiment of this aspect of the invention includes a cell containing a first exogenous gene encoding an immunopotentiating polypeptide and a second exogenous gene encoding a "lethal" or "suicide" polypeptide, preferably under the control of an inducible promoter. Introduction of the second exogenous gene confers the ability to selectively kill the tumor cell by inducing a promoter in operative linkage to the gene encoding the lethal polypeptide to initiate transcription of the polypeptide. Diphtheria toxin or the thymidine kinase of Herpes simplex virus are exemplary lethal genes. An exemplary promoter employed is the 6-16 promoter, which is inducible with low levels of interferon. Methods for using the cells in cancer therapy are also provided.

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METHODS AND COMPOSITIONS FOR GENETIC THERAPY
AND POTENTIATION OF ANTI-TUMOR IMMUNITY

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This invention relates generally to the field of
immunotherapy of cancer, and more specifically, to
preparation of tumor cell lines containing an exogenous
gene encoding a polypeptide that potentiates the immune
response to the tumor, for example, interleukin-2. The
invention includes methods for cancer therapy by
immunization with the immunopotentiating tumor cell.

The invention also provides methodology whereby one
can selectively ablate in vitro and in vivo genetically
altered cells. A preferred embodiment of this aspect of
the invention includes a cell containing a first exogenous
gene encoding an immunopotentiating polypeptide and a
second exogenous gene encoding a "lethal" or "suicide"
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of the polypeptide. Diphtheria toxin or the thymidine
kinase of Herpes simplex virus are exemplary lethal genes.
An exemplary promoter employed is the 6-16 promoter, which
is inducible with low levels of interferon. Methods for
using the cells in cancer therapy are also provided.

Active immunotherapy is considered to be a promising
approach to the treatment and particularly to the
prevention of recurrences of human cancer. Specific

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active immunotherapy, one of the most promising approaches
under investigation, involves activation of the host
immune response against the tumor by immunization with
tumor cells (which may be altered by mutagenesis, by
5   treatment with a hapten, or by expression of foreign
proteins) in order to activate specific effector cells of
the immune system, such as cytolytic T lymphocytes.
Nonspecific active immunotherapy may utilize microbial or
chemical immunomodulators to activate natural killer (NK)
10   cells, macrophages or lymphokine activated killing (LAK).
Unfortunately, much of the promise of these approaches
remains unfulfilled.

One of the most critical questions in cancer
immunology is why the immune system fails to eliminate
15   tumors. In the 1970's, Hewitt articulated the notion that
most tumors did not express any tumor-specific or
neoantigens and thus could not be recognized as "foreign"
by the immune system. Indeed, virtually no tumor cell
surface antigens recognized by antibodies were found to be
20   tumor specific, and furthermore, most spontaneous murine
tumors were considered "poorly immunogenic" as defined by
their failure to be eliminated when transferred into
syngeneic hosts (Hewitt et al., 1976). However, these
same tumors could be rendered "immunogenic" by mutagenesis
25   (Van Pel and Boon, 1982) when new antigens are expressed
on the tumor cell surface.

It is possible that the immune system fails to
eliminate tumors not because neoantigens are absent, but
rather because the response to these neoantigens is
30   inadequate. Therefore, a method for enhancing
immunogenicity of the tumor cells so as to potentiate the
host's immune response would provide a key advance in
immunotherapy.

Failure to respond to tumor neoantigens may be due,
35   at least in part, to a failure of T cell help. The
molecular basis for Th function is the local secretion of

lymphokines, such as interleukin-2 (IL-2), that act upon
CTLs whose T cell receptors have first been engaged by the
appropriate antigen-MHC complex (reviewed in Moller,
1980). The cytotoxic potential of NK and LAK cells is
also enhanced by IL-2 (Grimm et al., 1982; Phillips and
Lanier, 1986; Ortaldo et al., 1986). Although potentiation
of tumor immunity by systemic injection of interleukin 2
has been attempted, those studies were hampered by the
toxicity of the systemically administered IL-2.
Therefore, a method for potentiating immunity to tumors by
providing accessory T cell help in the location of the
tumor is a more attractive option, which has long been
needed for cancer therapy.

An additional difficulty in immunotherapy results
from the problems inherent in administration of a living
neoplastic cell to a patient. In the past, tumor cells
used for immunization were treated prior to immunization
to reduce their proliferative potential, e.g., by
irradiation or treatment with mitomycin C. Unfortunately,
either one of these methods of inhibiting replication also
significantly diminishes the immunogenicity of the cells.
It has been shown that mutagen induced variants that were
irradiated with 8-10,000 Rads are no longer immunogenic
(Sella, 1989; Boon, 1985). Similarly, murine tumor cells
secreting IL-2 of IFN-γ lose their immune potential after
irradiation. In addition, attempts at using membrane
preparations of tumor cells also fail to produce
convincing evidence of an immune response. It would
therefore be advantageous to develop a means for using
viable immunogenic tumor cells that could be deleted after
they have induced an immune response.

The present invention provides for a novel
immunopotentiating tumor cell variant, capable of inducing
a host immune response to tumor antigens, comprising a
selected immunopotentiating gene, for example,
interleukin-2. The invention also provides a variant

comprising both an immunopotentiating gene and a lethal
gene system.

In a first general embodiment, the invention provides
a cellular preparation for potentiating the immune
response to a tumor by a vertebrate organism, such as man
or other mammal, birds, or fish. The preparation
comprises cells, derived from the tumor, that contain an
exogenous gene (e.g., one introduced by insertion of
genetic material from outside the cell, for example, by
transfection and selection of a stably transfected cell
line) which encodes an immunopotentiating interleukin. In
a preferred embodiment, the interleukin is interleukin 2.
Although applicants are not bound by theory, evidence
suggests that the interleukin 2 gene confers upon the cell
the ability to induce a specific immune response, probably
mediated primarily by T lymphocytes.

In a related embodiment, the invention also provides
a composition for enhancing non-specific resistance to a
tumor, that embodiment including a preparation of cells
containing an exogenous gene encoding interferon,
preferably, interferon γ.

Although it is not necessary for practice of the
invention, various gene combinations may produce greatly
advantageous or even synergistic effects on potentiation
of immunity to the tumor. Therefore, the invention also
provides immunotherapeutic cells that may contain more
than one exogenous gene. For example, the cells may
contain either an interleukin gene or an interferon gene
or both and another gene encoding an yet another
immunopotentiating polypeptide.

The immunopotentiating polypeptide may be defined as
a polypeptide that enhances responsiveness of a host
immune system to a tumor present in the animal. At least
two categories of immunopotentiating genes can be used in
practice of the invention. A first category,
"immunopotentiating antigens," includes genes encoding a

variety of antigens alien to the animal, such as virus
envelope proteins, bacterial cell antigens, and other
antigens, capable of eliciting an immune response in an
animal immunized with a cell expressing such antigen.

5   That category of genes is referred to here as
"immunopotentiating antigens." Preferred species among
the category include, for example, viral envelope
proteins, e.g., the influenza hemagglutinin (HA) gene, the
influenza neuraminidase gene, vaccinia genes, or vesicular

10   stomatitis virus genes. Suitable bacterial antigens
include, for example, the 65kDa antigen of M. tuberculosis
and other bacterial antigens. Other immunopotentiating
genes may include those encoding histocompatibility
antigens allogenic to the host and other alloantigens.

15   A second category of immunopotentiating genes
includes genes encoding proteins that may not be
immunogenic to the host but nevertheless potentiate
immunity by activating or enhancing the activity of cells
of the immune system, such as T lymphocytes, natural

20   killer cells, or lymphokine activated killer cells.
Included in this category of immunopotentiating genes are
those encoding a number of the lymphokines classified as
"interleukins," and in particular, interleukin-2, -4, -5,-
6,-1, or -3. Also included are in this category, although

25   not necessarily working according to the same mechanism,
are proteins known as interferons, and in particular
interferon gamma.

         The genes may be obtained by techniques known in
molecular biology and introduced to a selected target cell

30   (which is usually derived from the tumor) by transfection
or by transformation with a vector suitable for use with
the particular target cell. Particular techniques
described by Sambrook, et al., Molecular Cloning, A
Laboratory Manual, Second Edition, Cold Spring Harbor

35   Press, 1989 (incorporated herein by reference) may
facilitate practice of certain aspects of the invention.

In one embodiment of the invention, one may employ a
population of selected target cells containing at least
one gene encoding an immunopotentiating antigen and a
second immunopotentiating gene selected from the second
5   category of immunopotentiating genes discussed above.

The invention also includes methods for potentiating
the immune response of a vertebrate organism to a tumor by
introducing into the organism a viable preparation of the
cells described here. In a preferred embodiment, the
10   cells are administered after reduction of tumor burden,
e.g., by surgical resection, irradiation, or other
appropriate techniques.

Of course, in many situations, it may be desirable to
selectively ablate or destroy the administered cells once
15   they have achieved the desired function. Therefore, in
another major embodiment of the invention, the cells
containing the immunopotentiating gene also contain a
"lethal gene" that allows one to selectively kill the cell
containing it should it become desirable to do so. In a
20   preferred embodiment, the lethal gene will be coupled to a
specifically inducible promoter, so that by treatment with
the inducer, one can specifically promote transcription of
the mRNA encoding the "lethal protein," which, in turn, is
capable of directly or indirectly killing the host cell,
25   for example, by inhibition of intracellular metabolism.

Therefore, according to this embodiment the invention
provides a cell, which comprises a first exogenous gene
encoding a selected polypeptide and a second exogenous
gene ("lethal gene") including a promoter, preferably a
30   selectively inducible promoter, in operative linkage to
DNA encoding a second selected polypeptide. The second
selected polypeptide, a "lethal polypeptide," is capable
of killing the cell, for example, upon induction of a
selectively inducible promoter or upon addition of a
35   substrate for the lethal polypeptide, which substrate is
converted by the lethal polypeptide to a molecule that is

toxic for the cell. By the term "operative linkage," it
is meant that the promoter is located in a position
relative to that of the DNA encoding the lethal
polypeptide that allows the promoter to effectively direct
transcription of the structural gene for the lethal
polypeptide.

In a more specific embodiment directed to cancer
immunotherapy, the invention provides a composition for
potentiating the immune response to a tumor, the
composition comprising a cell derived from the tumor,
which cell contains a first exogenous gene encoding an
immunopotentiating polypeptide and a second exogenous gene
including a promoter in operative linkage to DNA encoding
a polypeptide capable of killing the cell. Preferably,
the promoter will comprise a specifically inducible
promoter and the lethal polypeptide kills the cell upon
induction of the promoter.

Although any of a number of suitable promoters, such
as the SV40, cytomegalovirus, or actin promotors may find
utility in practice of this aspect of the invention, the
inducible 6-16 interferon α/β promoter described below is
particularly advantageous. Alternatively, when a gene
encoding a polypeptide which requires addition of an
exogenous substrate for intracellular toxicity is used
(such as the Herpes simplex thymidine kinase that requires
gancyclovir), a constitutive promoter such as the Herpes
simplex promoter may be employed.

A number of suitable lethal genes may be used.
Suitable examples include the Herpes simplex virus
thymidine kinase gene and the gene encoding Diphtheria
toxin A chain.

The cells containing immunopotentiating and lethal
genes may also include yet a third exogenous gene,
encoding a second immunopotentiating polypeptide. In a
preferred embodiment, the third exogenous gene encodes an
immunopotentiating antigen if the first immunopotentiating

polypeptide is a cytokine and encodes a cytokine if the
first immunopotentiating polypeptide is an antigen.

The invention also includes methods for producing the
novel cell derivatives. The invention includes, for

5   example, a method for producing a cell for therapy of a
selected disease comprising introducing a first exogenous
gene encoding a selected polypeptide into a cell and
introducing a second exogenous gene containing a promoter,
preferably a selectively inducible promoter, in operative

10 linkage with DNA encoding a polypeptide capable of killing
the cell, into the cell to produce a cell having both the
first and the second exogenous gene. Provision of a
selectively inducible promoter allows one to kill the cell
upon induction of the promoter. Cells, genes, and

15 promoters, and lethal genes as described above are
suitable for use with this aspect of the invention as
well.

Of course, the invention also includes methods for
use in gene therapy, and specifically, immunotherapy of

20 cancer. An exemplary method for gene therapy of a
vertebrate organism comprises introducing a first
exogenous gene encoding a selected polypeptide into a
tumor cell and introducing a second exogenous gene
containing a promoter in operative linkage with DNA

25 encoding a polypeptide capable of killing the cell into
the cell to produce a cell having both the first and the
second exogenous genes stably maintained in the cell; and
administering the cell, preferably by injection into a the
organism. In the preferred embodiment where the promoter

30 is a selectively inducible promoter, the additional step
of inducing the promoter to promote synthesis of the
lethal polypeptide may be performed when warranted.
Alternatively, when a constitutive promoter is used, e.g,
in conjunction with the Herpes simplex thymidine kinase

35 gene, administration of a second toxic agent, such as
gancyclovir, may comprise an additional step of the

methods. Of course, the Herpes simplex thymidine kinase gene may also be placed under control of a selectively inducible promoter, in which case both induction of the promoter and administration of the second agent may occur.

The methods may be used for immunotherapy of a variety of tumors, including, for example, melanoma, breast cancer, sarcoma, colon cancer, and ovarian cancer.

These and other aspects of the invention will become more apparent from a description of particular embodiments when read in conjunction with the drawings.

FIGURE 1

Induction of CTLs after In Vivo Injection of CT26 cells and IL-2-Transfected CT26-IL-2$^+$ Cells. Cells (1×10^6) of either the CT26 or CT26-IL-2$^+$ lines were injected subcutaneously into the left flank of BALB/c mice. After 2 weeks, splenocytes were removed and cultured 5 days with mitomycin .-treated CT26 cells in the presence of IL-2. At the end of culture, live cells were mixed with ^{51}Cr-labeled CT26 targets at different effector to target ratios in a 4 hr ^{51}Cr release assay. (a) CTLs generated from CT26 vs. CT26-IL-2$^+$ cells. (b) Anti-CD4 and anti-CD8 blocking of CT26 lysis by splenocytes from mice immunized with CT26-IL-2$^+$ cells. (c) Anti-MHC class I and class II blocking of CT26 lysis by splenocytes from mice immunized with CT26-IL-21$^+$ cells.

FIGURE 2

Protective Immunity against CT26 Cells Induced by Injection of CT26-IL-2$^+$ Cells. BALB/c mice were injected subcutaneously in the left flank with 1×10^6 CT26-IL-2$^+$ cells followed by subcutaneous challenge in the right flank with 1×10^5 CT26 cells either 2 weeks or 4 weeks later. Also shown is CT26 growth without CT26-IL-2$^+$ pre-immunization. Tumor growth was assessed every week by palpation and measurement. The pooled results of 20 mice

per group are shown. Data is presented as a Kaplan-Meier
plot.

FIGURE 3

5   Effects of T Cell Subset Depletion on the In Vivo Response
to CT26-IL-2$^+$ and CT26-HA$^+$ Cells. BALB/c mice were
depleted in vivo of either CD4$^+$, CD8$^+$, or both CD4$^+$ and
CD8$^+$ T cells by intraperitoneal injection of purified
anti-CD4 or anti-CD8. They were then injected
10   subcutaneously in the left flank with 1 x 10^6 CT26-neo-IL-
2$^+$ cells. Also shown is the growth in normal and CD4-
depleted BALB/c mice of a CT26-HA$^+$ line rendered
immunogenic by transfection with the influenza
hemagglutinin gene. Tumor growth was measured every week
15   and is presented as in Figure 2. Specific depletion of T
cell subsets was documented as described in Experimental
Procedures.

FIGURE 4

20   Fluorescence intensity distribution based on the binding
of labeled goat anti-mouse immunoglobulin to SP1 or CT-26
cells pre-treated with mouse anti-H2K^k or H2K^d,
respectively.

25   **FIGURE 5**

SP1 cells were treated with 10 U/ml of IFN$_\gamma$ for 24 hours.
The cells were washed and a sample was removed for FACS
analysis. The cells were returned to in vitro culture
without additional IFN$_\gamma$ and samples were removed for FACS
30   analysis at 48, 72 and 96 hours. This figure shows the
fluorescence intensity distribution based on binding of
goat anti-mouse immunoglobulin to IFN$_\gamma$ treated SP1 cells
pre-treated with mouse anti-H2K^k antibody.

FIGURE 6

Three distinct clones of SP1 cells transfected with the
murine IFN$_\gamma$ gene were assessed for H2K^k expression. The
figure demonstrates the fluorescence intensity

5   distribution based on the binding of goat anti-mouse
immunoglobulin to cells pre-treated with mouse anti-H2K^k.

FIGURE 7

A group of six nude mice were injected s.c. with 1 x 10^5

10 6L cells. Two of the six resulting tumors were removed,
cultured in vitro and assessed for IFN$_\gamma$ production and
H2K^k expression. The individual tumors 6L-A and 6L-B were
then injected into syngeneic mice and assessed for their
tumorigenicity.

15

The following examples illustrate selected aspects of
the invention but are not intended to limit it except as
specified in the claims.

20 **EXAMPLE I**

**Enhancement of Anti-Tumor Immunity by Immunization with
Cells Transfected with an Exogenous Gene Encoding
Interleukin 2.**

25   The following example describes results obtained upon
practice of one aspect of the invention--potentiation of
an anti-tumor immune response by administering cells
transfected with a gene encoding an exogenous
immunopotentiating cytokine, in this case, interleukin 2.

30

A.   **Experimental Procedures**
    1.   **Cells**
    CT26 cells were obtained from M. Brattain (Brattain
et al., 1980). The F10 subline of B16 melanoma cells

35 (Fidler, 1975) was obtained from the NIH DCT tumor
repository. RENCA is a murine renal cell carcinoma
originally described by Murphy and Haushesky (1973). SS-5

is a spontaneous mammary adenocanthoma induced by methylcholanthene in one of our laboratories (P.F.). YAC-1, an MHC⁻ NK target cell line (Kiessling et al., 1975), was kindly provided by J. Wagner, Johns Hopkins University.

2. Transfections

DNA was introduced into cells as a coprecipitate with calcium phosphate (Graham and van der Eb, 1973; Wigler et al., 1979). The CT26-IL-2$^+$ cell line was obtained by transfection with 5 μg of the plasmid vector pBCMG-neo-mIL-2, a bovine papilloma virus expression vector containing a murine IL-2 cDNA clone under the transcriptional control of a cytomegalovirus promoter with a rabbit β-globin intron, splice, and poly(A) addition signals; it also contains the Th5 neomycin-resistance gene (Karasuyama and Melchers, 1988; Karasuyama et al., 1989). Cells were exposed to the precipitate for 14-16 hr, washed once with Hanks' balanced salt solution without Ca^{2+} or Mg^{2+}, refed with Dulbecco's modified Eagle's medium with 10% FCS, and incubated at 37°C. Selection in G418 at 400 μg/ml was begun 48 hr after cells were exposed to precipitate. The CT26-neo-IL-2⁻ line was produced by transfection of CT26 cells with a plasmid derived from pBCMG-neo-IL-2 by removing the cytomegalovirus early promoter, rabbit β-globin intron, and mIL-2 sequences. The hemagglutinin-expressing CT26-HA$^+$ cell line (described in Fearon et al., 1988) was produced by cotransfection of CT26 with 5 μg of the plasmid vector pBV1-MTHA and pSV2-neo followed by G418 selection. FACS 3, clone 5 was used in the studies here. The B16-IL-2$^+$ transfectant was generated similarly to the CT26-IL-2$^+$ transfectant but was cloned by limiting dilution after G418 selection.

3. IL-2 Assays

Supernatants of transfected cells were assayed for
IL-2 as previously described (Janis et al., 1989) by
transferring dilutions to 96-well microtiter plates
containing 3000 CTLL-2 cells per well. After 24 hr,
[^{3}H]thymidine was added for 12 hr, after which
incorporation was assessed with a PHD cell harvester.
Units per milliliter IL-2 was calculated as the reciprocal
of the supernatant dilution giving half-maximal
proliferation of CTLL-2.

4. CTL Assays

For CTL assays, spleens were removed from BALB/c mice
2 weeks after subcutaneous injection in the left flank of
1 x 10^6 CT26 or CT26-IL-2$^+$ cells. Mitomycin C treatment
of CT26 cells was performed by incubating them in 50 μg/ml
mitomycin C for 45 min at 37°C followed by three washes
with RPMI-10 FCS. In vitro stimulations were performed in
24-well plates for 5 days with 2 x 10^5 CT26 stimulators
and 6 x 10^6 splenocyte responders per well plus 20-50 U/ml
recombinant murine IL-2. ^{51}Cr release assays were
performed by mixing various numbers of effector cells with
5000 ^{51}Cr-labeled targets per well in 96-well V-bottom
plates. After 4 hr at 37°C, 100 μl per well was removed
and counted in a gamma counter. Percent specific lysis
($[cpm_{exp} - cpm_{min}]/[cpm_{max} - cpm_{min}]$ x 100) is plotted on the
y-axis for various effector to target ratios. CTL assays
for the B16 melanoma system were performed in a similar
fashion, except that incubations with ^{51}Cr-labeled B16
targets were performed for 8 hr. For antibody blocking, a
1:100 dilution of ammonium sulate-purified preparations
of the following antibodies were added to microwells at
the start of ^{51}Cr release assays: GK1,5, monoclonal
antibody (MAb) to CD4 (Dialynis et al., 19830; 2.43, MAb
to CD8.2 (Sarmiento et al., 1980); M5-114, MAb to I-A^d +
1-E^d (Battacharaya et al., 1981); 28-14-8, MAb to L^d (Ozato

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et al., 1980); 35-1-2, MAb to K^d + D^d (Ozato et al., 1982).
All blocking antibodies were carefully titered such that
the concentrations used were 5-10 times the minimal
concentration that yielded saturation binding to splenic
5   lymphocytes as assayed by flow cytometric analysis of
serially diluted antibody. Prior to use, blocking
specificity was assessed as follows: anti-CD4 and anti-
MHC class II antibodies inhibited in vitro secondary PPD-
proliferative responses by more than 80% and allo-CTL
10   lysis (B6 anti-BALB/c) by more than 5%; anti-CD8 and anti-
MHC class I antibodies inhibited secondary PPD-
proliferative responses by more than 5% and allo-CTL lysis
by more than 80%.

15   5.   In Vivo Antibody Depletions
             In vivo antibody depletions were started 1-2 days
prior to injection of the tumor. MAb GK1.5 was used for
CD4 depletions and MAb 2.43 was used for CD8 depletions.
Ammonium sulfate-purified ascites fluid (titered at
20   >1:2000 by staining of thymocytes on the FACS) was
injected intraperitoneally (0.1 ml per mouse) every other
day for the first 3 weeks and then once per week
afterward. Depletion of T cell subsets was assessed on
the day of tumor injections, 3 weeks, and 5 weeks after
25   tumor injection by flow cytometric analysis of lymph node
cells stained with 2.43 or GK1.5, followed by fluorescein
isothiocyanate-labeled goat antibody to rat IgG. For each
time point of analysis, >99% depletion of the appropriate
subset was achieved with normal levels of the opposite
30   subset present (in the case of the single depletions).

B.   Results
     1.   Production of IL-2 Transfected CT26 Cells
          The N-nitroso-N-methylurethane-induced murine colon
35   tumor line CT26 chosen for study is poorly immunogenic; a
small number of cells (1×10^3-1×10^4) injected into

syngeneic (BALB/c) mice cause a lethal tumor and do not
induce detectable tumor-specific CTLs (Fearon et al.,
1988). CT26 cells were transfected with a bovine
papilloma virus (BPV) vector containing a neomycin-
resistance gene and a murine IL-2 cDNA. Transfectants
were selected in the neomycin analog G418, and a G418-
resistant line (CT26-IL-2$^+$) prepared from more than 50
pooled clones of approximately equal size was chosen for
further study.

To assay for IL-2 production, the CT26-IL-2$^+$ line was
plated at 5×10^4 cells per well in a 24-well plate, and
after 3 days, supernatants (1.5 ml per well) were removed
and transferred in serial dilutions to the IL-2-dependent
CTLL-2 cell line. They were found to contain 40 U of IL-2
activity (1 U defined as induction of 50% maximal CTLL
stimulation in a 24 hr assay), indicating that CT26-IL-2$^+$
cells secreted significant quantities of IL-2. No
detectable IL-2 activity was found in supernatants from
either the parental CT26 cells or CT26 cells transfected
with the BCMG vector that had the IL-2 cDNA insert cut
out.

2. <u>CTL Generation Induced by CT26-IL-2$^+$ Cells</u>

Subcutaneous injection of CT26 cells has been shown
previously to elicit little if any detectable systemic CTL
activity, even after a secondary in vitro stimulation in
the presence of IL-2 (Fearon et al., 1988). However,
after subcutaneous injection of the IL-2-producing CT26-
IL-2$^+$ cells, significant anti-CT26 CTL activity was
detected in the spleen after secondary in vitro
stimulation (Figure 1a). The majority of in vitro CTL
activity was blocked by antibodies to CD8 and to MHC class
I, but not by antibodies to CD4 or to MHC class II
(Figures 1b and 1c). This suggested that CT26-IL-2$^+$ cells
indeed activated endogenous, MHC class I-restricted CD8$^+$
CTLs. Virtually no cytolysis was observed against another

BALB/c-derived tumor target (SS-5) or the MHC class I⁻ NK
target, YAC-1 (<5% specific lysis at an effector to target
ration of 100:1). These results demonstrate that most of
the effector cells induced by CT26-IL-2⁺ immunization were
5   antigen- and MHC class I-specific and that NK and LAK
cells represent a minority of the activity measured in
vitro.

     3.   In Vivo Immune Response Induced by CT26-IL-2⁺
10   Cells
     We next sought to determine whether the CTL response
elicited by CT26-IL-2⁺ cells correlated with in vivo
immunity. Following even the largest subcutaneous
injection of up to 1 x 10⁶ CT26-IL-2⁺ cells, no tumors
15   were seen up to 8 weeks after injection. In contrast,
tumors were present in all animals following injection of
the parental CT26 cells by 2 weeks (Table 1). Thus,
BALB/c mice were capable of rejecting 3-4 orders of
magnitude greater numbers of CT26-IL-2⁺ cells than the
20   parental CT26 cells. To demonstrate that rejection of
CT26-IL-2⁺ cells is due to activation of CT26-specific
effector cells by the locally produced IL-2, CT26 cells
were transfected with the entire BPV vector sequences
alone with the IL-2 insert removed. These transfectants
25   (CT26-neo-IL-2⁻), which were confirmed not to secrete IL-2
by CTLL functional assay, produced tumors in BALB/c mice
at a rate indistinguishable from the nontransfected CT26
cells (Table 1). In addition, we observed that injection
of 1 x 10⁶ CT26-IL-2⁺ cells on the left flank did not
30   inhibit the growth of 1 x 10⁵ CT26 cells on the opposite
flank. Thus, the effect of IL-2 production by CT26-IL-2⁺
cells appeared initially to stimulate a local, rather than
systemic, immune response.
     Given the result that immunization with CT26-IL-2⁺
35   cells induced systemic CTLs after two weeks, as measured
in vitro, we sought to determine whether this correlated

with an in vivo antitumor response against the parental
CT26 cells. Indeed, injection of CT26-IL-2$^+$ cells
completely protected mice against a challenge with 1 x 10^5
CT26 cells 2 weeks later (Figure 2). This protection was
not long-lived, as roughly 50% of mice challenged 4 weeks
after immunization developed tumors (Figure 2). The
protection was tumor specific in that other BALB/c tumors
(SS-5, RENCA) grew normally when injected 2 weeks after
CT26-IL-2$^+$ immunization (data not shown).

        4.   CT26-IL-2$^+$ Cells Bypass CD4$^+$ T Helper Function
        Because most T cell helper function is performed by
the CD4$^+$8$^-$ subset of T lymphocytes and most MHC-restricted
CTL function is performed by the CD4$^-$8$^+$ subset, we next
investigated the effect of selective in vivo depletion of
these subsets on the rejection of CT26-IL-2$^+$ cells.
Figure 3 shows that mice depleted of CD4$^+$8$^-$ T cells were
fully capable of rejecting CT26-IL-2$^+$ cells. Previous
studies have shown that CT26 cells could be rendered
immunogenic by transfection with a foreign gene such as
influenza hemagglutinin (CT26-HA$^+$; Fearon et al., 1988).
The "failure of help" hypothesis suggested that the
enhanced immune response induced by CT26-HA$^+$ cells might
be mediated, at least in part, by CD4$^+$ helper T cells
responding to MHC class II-restricted epitopes on the
exogenously introduced hemagglutinin gene product. In
support of this hypothesis is the observation that CT26-
HA$^+$ cells failed to be rejected by CD4-depleted mice
(Figure 3). Taken together, these results support the
concept that CT26-IL-2$^+$ immunization effectively bypasses
Th function in the generation of an antitumor CTL
response.

5.   CD8^{+} Cells Are Required In Vivo For Tumor
      Rejection

          Mice depleted of CD4[-]8^{+} cells were incapable of
rejecting CT26-IL-2^{+} cells, indicating that this T cell
5   subset is involved in the antitumor response in vivo
(Figure 3). It is noteworthy, however, that there was a
significant lag in the tumor's growth kinetics relative to
the kinetics of CT26 growth in normal BALB/c mice. While
this partial response could be due to the action of
10   residual CD4[-]8^{+} cells, none were detected in lymph nodes
by flow cytometric analysis either at the time of initial
tumor injection or during the course of in vivo antibody
treatment. A similar delay in tumor growth was seen in
the presence of both CD4 and CD8 depletion. It is
15   therefore likely that an IL-2-responsive, CD4[-]8^{-} effector
cell population was additionally involved in the in vivo
antitumor response. Three IL-2-responsive candidates are
NK cells, LAK cells, and T cells bearing the $\gamma\delta$ T cell
receptor (reviewed in Pardoll et al., 1987; Brenner et
20   al., 1988; Raulet, 1989). As most of the cells in these
populations are CD4[-]8^{-}, they would still be present in CD8-
depleted mice. Other potential effector cells involved in
the antitumor response, based on histologic analysis of
regressing tumors, include macrophages and mast cells
25   (data not shown).

6.   CTL Generation and In Vivo Immunity Induced by
      IL-2-Producing Melanoma Cells

          To assess whether the induction of systemic immunity
30   was a general feature of tumors engineered to secrete IL-
2, we transfected a second poorly immunogenic tumor, B16
melanoma, with the BCMG-IL-2 vector. The B16 melanoma is
a highly aggressive melanocyte tumor of C57BL/6 origin.
After transfection, G418-resistant clones were chosen
35   (B16-IL-2^{+}) that produced quantities of bioactive IL-2
equal to or greater than cells, when assayed under

identical conditions on CTLL cells. Table 2 shows the results of a representative clone. While small numbers of B16 melanoma cells caused tumors in syngeneic C57BL/6 mice, B16-IL-2$^+$ cells were completely rejected. As with the CT26 tumor, greater CTL activity was generated against the parental B16 tumor in mice injected with B16-IL-2$^+$ cells. C57BL/6 mice injected with B16-IL-2$^+$ cells developed protective immunity in vivo against challenge with B16 cells in a dose-dependent manner. The protection was not as great as seen in the CT26 system, as roughly half the mice immunized with 1×10^6 B16-IL-2$^+$ cells did eventually develop tumors after challenge with 1×10^5 parental B16 cells (Table 2).

It is noteworthy that MHC class I expression on B16 melanoma cells is extremely low - roughly 5- to 10-fold lower than on CT26 cells (data not shown). This may account for the apparently lower efficiency of protection against challenge with parental tumor in the B16 than in the CT26 system, although the faster growth rate of B16 melanoma cells may be an additional contributing factor.

In addition to the colon tumor and melanoma data presented here, similar results have been recently observed with an IL-2-transfected murine CBA-SP1 sarcoma and the rat Dunning prostate carcinoma (data not shown). The fact that similar effects were seen with two tumors of widely differing cellular origins suggests that the principles outlined here may be generalizable to a variety of cancers. In other studies, however, a spontaneous mammary adenocarcinoma derived from CBA mice was transfected with the IL-2 gene and used to immunize CBA mice against growth of the parental tumor. In those studies, the IL-2$^+$ transfectant did not induce a significant immunity against the parental tumor.

We have also prepared a number of additional transfectants expressing HA and/or secreting IL-2 or IFN-

γ. It is believed that the dually transfected cells
should improve the immunogenic status of the tumor.

EXAMPLE II
5   Production of Tumor Cells Transfected with an Exogenous
Gene Encoding Gamma Interferon and Demonstration of
<u>Enhanced Non-Specific Tumor Resistance</u>

        The following example describes yet another aspect of
the invention, transfection of cells with a gene encoding
10   a cytokine capable of non-specifically enhancing tumor
resistance, probably through a mechanism dependent on
cells other than T lymphocytes. In the following example,
the non-specific enhancement of tumor resistance was
produced by injecting mice with a tumor cell transfected
15   with the gene encoding gamma interferon.

        More specifically, SP1 murine adenocarcinoma cells
transfected with the murine IFN_γ gene expressed IFN_γ
($SP1/IFN_\gamma$), failed to grow in syngeneic hosts, and grew in
nude mice. The rejection of $SP1/IFN_\gamma$ cells was related to
20   the amount of IFN_γ produced and appeared to be mediated
primarily by nonspecific cellular mechanisms, though some
role for T cells in the afferent arm of this response is
possible. SP1 are $H2K^k$ negative but express class I
antigens when producing IFN_γ. However, class I MHC
25   expression, while likely necessary, was insufficient in
itself to prevent tumor growth since secretion of >64 U/ml
IFN_γ was needed to inhibit tumorigenicity while only 8
U/ml IFN_γ could induce class I antigens. Similar results
were obtained with the murine colon carcinoma CT-26, a
30   tumor that constitutively expresses class I MHC antigens,
further supporting the contention that Class I MHC
expression is not essential for the rejection response
induced by IFN_γ. The failure of $SP1/IFN_\gamma$ cells to protect
against a challenge with parent SP1 cells argues that
35   factors other than IFN_γ production or class I MHC

expression are needed to induce a protective response
against weakly or non-immunogenic tumor cells.

A.   Materials and Methods
5   1.   Mice.
          CBA and BALB/c female mice between 6 and 8 weeks of
age were obtained from the Frederick Animal Facility of
the National Cancer Institute.

10   2.   Tumors.
          The origin of the SP1 spontaneous mammary
adenocarcinoma has been described previously (Frost et
al., 1987). SP1 grows readily in syngeneic mice after
s.c. inoculation and is poorly, if at all, immunogenic and
15 expresses little if any class I MHC antigen. The CT-26
colon adenocarcinoma was obtained from M. Brattain
(Brattain et al., 1980). MDW1 is an immunogenic variant
of the MDAY-D2 lymphoma (Kerbel, 1979).

20   3.   Culture Conditions.
          Cells were cultured in GIBCO RPMI 164C medium
supplemented with 50,000 U of penicillin and streptomycin,
150 mg of L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-
piperazine ethanesulfonic acid (HEPES), 375 mg of sodium
25 bicarbonate, and 10% fetal calf serum (Irvine Scientific,
Santa Ana, CA). All tumors were tested periodically for
the presence of *Mycoplasma* using the Gen Probe RNA
hybridization method (Gen Probe, San Diego, CA);
contamination with *Mycoplasma* was never observed. In
30 addition, the cells were tested for murine antibody
production (Microbiological Ass iates, Bethesda, MD) and
found to be free of 13 murine pathogenic viruses.

          4.   Antisera.
35   The monoclonal antibodies used in these studies were
as follows: Murine anti-D^k/K^d (clone 15-5-5S) was kindly

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provided by Dr. B.E. Elliott (Queen's University, Kingston, Ontario, Canada). Murine anti-K^d (clone 16-1-11N) was purchased from the Litton Company (Charles, SC). Murine anti-K^k/D^k Ab (clone H 100-27/55), anti-I-A^k (clone 14V18), and anti-D^k were purchased from the Cedarlane Company (Ontario, Canada). Murine anti-I-A^d antisera was obtained from Becton Dickinson (San Jose, CA).

5.   In Vitro IFN$_\gamma$ Treatment.

Recombinant murine IFN$_\gamma$ was obtained from Genentech, Inc. (San Francisco, CA). 3×10^5 tumor cells were plated in five 10-cm plastic dishes and incubated with 1-100 U/ml of IFN$_\gamma$ for 4 days. On the fourth day, IFN$_\gamma$-treated cells were aliquoted and reincubated with 1-100 U/ml of IFN$_\gamma$ for an additional 3 d. The cells were then analyzed for the expression of class I or class II MHC antigens.

6.   Flow Cytofluorometry.

Quantitative analysis of MHC expression on cell surfaces were performed using FACS. Cells were scraped from tissue culture plates using a rubber policeman and incubated with murine anti D^k, D^d or IA^k or IA^d antibodies for 30 min at 4°C. After being washed, the cells were incubated with FITC-conjugated goat anti-mouse IgG antibodies at 4°C for 30 min, fixed with 1% paraformaldehyde and examined within one week using cytofluorometry.

7.   Transfection of Cells With DNA.

DNA was introduced into SP1 or CT-26 cells either as a coprecipitate with calcium phosphate (Graham and van der Eb, 1973) or via use of the lipofectin reagent (Felgner et al., 1987). The SP1-neo and CT-26/neo cell lines were obtained by transfection with a mixture of 1 ug of the plasmid vector pSV2neo with 1 ug of mouse liver DNA. The SP1-IFN$_\gamma$ lines were obtained by transfecting SP1 cells

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with 1 ug of pSV2neo and 10 ug of pGEM3-SVMu IFN$_\gamma$. The
latter is a pGEM vector containing an SV40 promoter and
the murine IFN$_\gamma$ gene and was kindly provided by Dr. R.
Suzuki of the Department of Immunology at the M.D.

5   Anderson Cancer Center. After selection in geneticin, the
colonies form individual plates were pooled and assessed
for IFN$_\gamma$ production.

        8.   Anti-Viral Assay for Interferon Activity.
10   The method of Mory et al., was used (Mory et al.,
1981) to assay IFN activity. This assay relies on the
inhibition of vesicular stomatitis virus growth in murine
LTK cells. Control wells were treated with recombinant
IFN$_\gamma$ or medium alone. Supernatants from the experimental

15   cell cultures were added at doubling dilutions and their
ability to inhibit VSV viral growth assessed by
determining how many wells contained viable LTK cells.

        9.   Assessment of Cell-Mediated Cytotoxicity.
20   Cytotoxicity was measured using the previously
described ^{111}In-release assay (Wiltrout et al., 1978).

        10.   Separation of Spleen Cells.
        Nylon wool adherent (NWA) and nonadherent cells
25   (NWNA) were obtained by the method described in Julius et
al., (1973).

B.   Results
        Prior to the transfection studies, we ascertained
30   whether the SP1 cells would express MHC class I antigens
in response to IFN$_\gamma$. In addition, we determined if the
addition of exogenous IFN$_\gamma$ had a cytostatic effect on
either the SP1 or CT-26 cell lines. One to 1000 U of IFN$_\gamma$
were added to semiconfluent cultures of SP1 or CT-26 cells

35   for four days. Class I MHC expression was then measured
and cell viability and replication was measured each day

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for five days and compared to non-IFN$_\gamma$-treated cells. CP1
cells grew normally in as much as 100 U/ml IFN$_\gamma$; but the
growth of CT-26 cells was inhibited by 17% in 1000 U/ml
IFN$_\gamma$. The growth rates of SP1 cells transfected and
5   secreting 256 U/ml IFN$_\gamma$ (6L) were the same as that of the
parent SP1 cells. Similarly, CT-26 cells producing 64
U/ml IFN$_\gamma$ had the same growth rate as parent CT-26 cells.

Figure 4 demonstrates the expression of H2K^k in Sp1
cells as well as H2K^d in CT-26 cells as assessed by FACS
10   using specific murine monoclonal antibodies. Fewer than
6-7% Sp1 cells expressed H2K^k, but all expressed IA^k (data
not shown). In contrast CT-26 cells expressed both class
I and II MHC antigens (Class II data not shown). After
treatment of SP1 cells with exogenous IFN$_\gamma$ the expression
15   of class II antigens did not change but a dramatic shift
of the FACS curve to the right was readily detectable with
anti K^k antibodies. IFN$_\gamma$ treatment had no effect on the
constitutively expressed class I or II antigens of CT-26
cells.

20   We next determined the duration of the effect of
exogenous IFN$_\gamma$ on class I expression by SP1 cells. These
experiments served as a basis for the use of transfection
with the IFN$_\gamma$ gene as a means for providing sustained
production of IFN$_\gamma$ and expression of class I MHC antigens.
25   SP1 cells were treated with 100 u/ml of IFN$_\gamma$ for three
days. The cells were washed and either assessed for H2K^k
expression or recultured. This was repeated at 24, 48, 72
and 96 hours after the removal of IFN$_\gamma$ from the medium.
Figure 5 shows that the H2K^k expression returned to
30   baseline levels within 48 hours after the removal of IFN$_\gamma$
from the medium. It is reasonable to assume that a
similar reduction in H2 expression would occur if IFN$_\gamma$-
treated cells were injected in vivo.

1.   **Effect of IFN$_\gamma$ Transfection on Class I MHC Expression.**

After transfection, both the SP1 and CT-26 lines were tested for the secretion of IFN$_\gamma$. Three SP1-IFN$_\gamma$ cell populations--1C, 3C and 6L-- were selected for study based on the amount of IFN$_\gamma$ secreted. The results are shown in Table 3. The CT-26/IFN$_\gamma$ cells were similarly shown to produce IFN$_\gamma$, but to a lesser degree than the SP1/IFN$_\gamma$ cells (8 U/ml vs 256 U/ml). Of interest is the demonstration in all three SP1/IFN$_\gamma$ cell lines of an equivalent increase in MHC class I expression (Figure 6, the results for the CT-26 cells are shown below)). In addition, all three lines expressed class II MHC antigens (IA^k) at a level comparable to the nontransfected parent cells (data not shown). SP1 cells transfected with the neo^R gene alone did not express class I MHC antigens.

2.   **Effect of IFN$_\gamma$ Gene Transfection on Tumor Growth In Vivo.**

SP1/IFN$_\gamma$ were injected s.c. into CBA mice. Table 3 summarizes the data from several such experiments. These cell lines were selected because they all demonstrated a significant level of H2K^k expression (Figure 6), when compared to five additional populations that were screened and found not to express H2K^k and were thus presumed not to be producing IFN$_\gamma$. 1C and 3C grew at challenge doses of 1×10^5. In contrast, 6L cells failed to grow in 14 of 15 animals injected with 1×10^6 cells; the parent SP1 cell line grew when as few as 10^3 cells were injected s.c. Immunogenicity correlated with the level of IFN$_\gamma$ production rather than H2K^k expression (Table 3 and Figure 6) since all three lines expressed equivalent levels of MHC antigen but only the 1C and 3C cell lines grew vigorously. 6L cells did grow in nude mice. Sp1 cells transfected with the neo^R gene alone grew in a manner

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identical to nontransfected SP1 cells (Kerbel et al., 1987).

3.   Nonspecific Cytotoxic Response to 6L.

5   Table 4 shows that viable 6L cells induced a
cytotoxic response reactive with itself and the parent SP1
tumors. This response was mediated by nylon wool adherent
(NWA) spleen cells but not by nylon wool non-adherent
(NWNA) cells. The cytotoxicity of the NWA spleen cells
10   against both SP1 and 6L was always greater than that
produced by the whole spleen cell population. In
addition, NWA cells were reactive (though to a lesser
degree) against the unrelated MDW1 tumor cell line. These
results implied that a non-specific mechanism was
15   responsible for the rejection of 6L cells. This view was
supported by our failure to diminish the splenic cytotoxic
response after depletion of immune spleen cells with anti-
CD4, anti-CD8 and anti-CD3 antibodies plus complement
(data not shown).
20   To further assess the nonspecific nature of the
rejection of 6L cells, 1×10^6 6L cells were mixed with 1
$\times 10^5$ SP1 cells and injected together into the left flank
of CBA mice. Five of 10 mice developed tumors at 5 weeks.
Syngeneic CBA mice injected with 1×10^4 SP1 cells develop
25   tumors within 2 weeks. The five tumors that grew were
removed and placed in tissue culture, allowed to grow for
two days, and then exposed to geneticin. In all cases,
the cells placed in culture failed to grow in the presence
of geneticin, indicating that these were parent SP1 cells,
30   which are sensitive to 500 ug/ml geneticin (6L cells carry
the neo^R originally used in the co-transfection with the
IFN$_\gamma$ gene). The fact that the growth of 6L mixed with SP1
cells was markedly impaired argues that nonspecific
mechanisms induced by the 6L cells were responsible in
35   part for its failure to grow in vivo.

This conclusion was indirectly supported by
experiments in which immunization of CBA mice with 6L
failed to protect animals against a significant challenge
with parental SP1 cells (Table 5). Fifty percent of
5   immunized animals were protected against a challenge with
1 x 10^4 cells injected in the opposite flank. However, as
few as 5 x 10^4 SP1 cells induced tumors in 100% of the
immunized animals. In addition, the injection of animals
simultaneously in the left flank with 6L and right flank
10   with SP1 had no effect on the growth of the SP1 cells,
though the 6L cells failed to grow.

4.   Role of Class I MHC Antigen Expression.

It remained to be determined whether the effect of
15   IFN_γ production was strictly due to its enhancement of
class I MHC expression or if IFN_γ produced locally had
other effects on the host. 6L cells were injected into 6
nude mice and produced tumors. Two of these tumors were
removed (6L-A and 6L-B) and recultured. These cell lines
20   were then assessed for IFN_γ production and H2 expression.
Figure 7 shows that 6L-A and 6L-B had equivalent
expression of $H2K^k$ even though 6L-B produced 64x more
IFN_γ. The reinjection of 6L-A and 6L-B into groups of six
CBA mice demonstrated that low IFN_γ-producing 6L-A cells
25   grew in all animals despite expressing $H2K^k$. In contrast,
high IFN_γ producing 6L-B cells failed to grow.

5.   The CT-26 Tumor.

We next determined the effect of IFN_γ expression of
30   murine tumor cells that constitutively express class I MHC
antigens. CT-26 murine adenocarcinoma cells were
transfected with the pGEM-SVMu-IFN_γ plasmid and the
original pool of transfected cells was found to secrete 8
U/ml of IFN_γ. Table 6 shows that at doses of 1 x 10^5 s.c.
35   or 5 x 10^4 i.v., CT-26/IFN_γ cells eventually grew, but
considerably more slowly than parent CT-26 cells.

The failure of CT-26/IFN$_\gamma$ cells to grow in vivo was directly related to their IFN$_\gamma$ production and could not be explained by any direct cytopathic effect of IFN$_\gamma$ because 8 U/ml IFN$_\gamma$ had no effect on CT-26 growth in vitro. In addition to reduced growth in vivo, CT-26/IFN$_\gamma$ induced a cytotoxic response detectable in the spleens of immunized mice (data not shown). Parent CT-26 cells have never been shown to engender any detectable cytotoxic response.

EXAMPLE III

Production of Immunopotentiating Tumor Cells
Co-Transfected With an Exogenous Gene Encoding a
Selected Immunopotentiating Polypeptide and a
Second Exogenous Gene Encoding a Polypeptide Capable
of Ablating the Host Cell on Specific Induction.

The following example describes yet another aspect of the present invention, the production of an immunogenic tumor cell that may be selectively ablated in circumstances when it becomes desirable to do so. In this aspect of the invention, cells are transfected with a selected gene encoding a desired polypeptide, e.g., the immunopotentiating polypeptide, and a second gene that encodes a polypeptide capable of ablating (killing) the host cell. The second gene, referred to as the "ablation gene" or the "lethal gene," is preferably under the control of a specifically inducible promoter. Thus, when it is desired to eliminate some or all of the transfected cells from the host (for example, at about 14 days after administration of the transfected tumor cells), one may initiate such elimination by directly or indirectly activating the inducible promoter to stimulate the synthesis of the lethal polypeptide, for example, by administering to the host an amount of the inducer sufficient to initiate transcription of the polypeptide. Specific examples of embodiments of this aspect of the invention are described below.

A.   Selection and transfection of genes encoding
     immunopotentiating polypeptides

Selection of the most advantageous immunopotentiating
polypeptide for use in a given situation will likely

5   depend on a number of parameters, including, for example,
tumor type, location, and immune status of the patient.
Genes encoding such polypeptides are either currently
available, or may be obtained using present techniques in
molecular biology, for example, by using techniques

10 described by Sambrook et al., in Molecular Cloning, A
Laboratory Manual, Cold Spring Harbor Press, 1989 (which
is incorporated herein by reference). Transfection of the
target cell type may generally be carried out essentially
according to the protocol set forth in example I above.

15 In the examples discussed more specifically below, the
genes selected for transfection are those encoding the
immunopotentiating polypeptides, IL-2, interferon gamma,
and influenza hemagglutinin.

20 B.   Selection and transfection with a specifically
     inducible lethal gene system.

     1.   Selection of a suitable promoter

Although a number of promoters, e.g.,
25 cytomegalovirus, SV40, or actin and the like could
conceivably be used in accordance with the invention, we
have selected the interferon-inducible 6-16 promoter for
use in a preferred embodiment. Use of this promoter has a
number of advantages because the levels of IFN in normal

30 blood are very low. Therefore, the injection of IFN can
trigger this gene in a very specific manner.

The human IFN-regulated gene 6-16 is tightly
controlled by IFN α/β. The 0.9 Kb cDNA of 6-16 encodes
for a 13 kilodalton protein which contains a signal

35 peptide sequence at the amino terminal end; however,
neither the function or the subcellular localization of
the protein (secreted or membrane bound) has been defined

(Kelly, 1986). Untreated cells have no detectable levels of 6-16 mRNA in their cytoplasm or nuclei. 6-16 mRNA synthesis can be detected as early as 5 minutes following the interferon alpha treatment; its synthesis in the nucleus of the cell peaks at three hours and decreases to basal levels within 8-12 hours (Friedman, 1984, Kelly, 1986). However, the mRNA of 6-16 is post-transcriptionally regulated and its presence in the cytoplasm can be detected even 12 hours after the nuclear synthesis has ceased (Friedman, 1984). The tight regulation of the 6-16 promoter has been used as a tool to obtain IFN-resistant mutants by linking the 6-16 promoter to selectable marker genes that also allow for back selection in case of low level deregulated expression (Pellegrini, 1989).

We have cloned and characterized the promoter region of 6-16 (Porter, 1988), its cis-acting IFN-regulatory sequences and the trans-acting factors with which they interact by 5' deletion analysis, transfection experiments, DNase-footprinting, gel retardation, in vitro transcription, and linking the promoter to reporter genes (Dale, 1989, Porter, 1988), (Chernajovsky, 1989).

The 6-16 promoter contains a direct repeat of 40 nucleotides (from -168 to -89) which includes the IFN-regulatory sequence that can confer IFN inducibility to heterologous promoters (Chernajovsky, 1989, Porter, 1988). This region binds a nuclear protein(s) of a molecular weight of 55 kilodaltons and can form in vitro a DNA-protein complex which is IFN modulated (Chernajovsky, 1990).

Another cis-acting element located at -450 binds constitutively to a nuclear protein of 80 kilodaltons and acts to down regulate expression of this promoter in conjunction with the direct repeat. By itself, this element seems not able to exert any transcriptional function.

In addition, the promoter of 6-16 has a CCAAT box
located in the non coding strand of the promoter and a
TATA box (Porter, 1988)(Chernajovsky, 1989)(Chernajovsky,
1990).

5

2.   Selection of Lethal Genes

Lethal genes that may be used in accordance with the
invention include genes encoding a variety of proteins
that inhibit or arrest intracellular metabolism, and in
10   particular, those leading to inhibition of key cellular
functions such as DNA synthesis, transcription and
translation, or those that paralyze other necessary
cellular functions. Exemplary lethal genes that may prove
useful in accordance with the invention include for
15   example, the genes encoding ricin beta chain, pertussis
toxin, etc. As discussed below, selection of a particular
lethal gene will often be governed by the physiologic
characteristics of the cell to which it will be
introduced.

20   For some rapidly growing tumors or cells, an agent
that will specifically inhibit DNA synthesis would offer
an advantage. However, for others, e.g., solid tumors
that grow slowly and do not proliferate very actively,
such a DNA synthesis inhibitor may be less useful. In
25   such cases, an agent that can specifically block another
metabolic function of the cell, such as protein
translation, could prove more relevant. Examples of
lethal gene systems designed to address each of those cell
types are discussed below.

30   For DNA synthesis inhibition, a preferred gene
encodes the enzyme thymidine kinase from Herpes simplex
virus. That enzyme is able to actively phosphorylate the
thymidine analog gancyclovir (GANC) (1-(-deoxy-2-fluoro-β-
D-arabinofuranosyl) -5-ethyluracyl), which after
35   conversion to the 5' triphosphate by cellular kinases,
inhibits DNA polymerase (Mansour, 1988, Mansuri, 1987).

The basis for the discriminating effect of GANC on cells
expressing HSV-tk and the cellular tk enzyme, is the fact
that the substrate requirements of the HSV-tk protein are
less stringent than those of the cellular tk. Thus, cells

5   expressing HSV-tk are sensitive to the toxic effects of
GANC; normal cells are spared.

   For protein synthesis inhibition, a preferred gene is
that encoding the α chain of Diphtheria toxin (DT-A). DT-
A ADP-ribosylates elongation factor 2, producing protein

10   synthesis inhibition and cell death. This gene has been
used successfully in transgenic mice to specifically
ablate pancreatic acinar cells when linked to the
enhancer/promoter of the elastase I gene (Palmiter, 1987).

15   3.   Construction of Selectively Inducible Lethal
            Gene Constructs

   The following prophetic examples describe
construction of genetic constructs suitable for use in
transfection of selected cells in accordance with the

20   invention.

         a.   Construction of a Herpes Simplex virus
             tk gene driven by the 6-16 promoter

   A Bgl II-Pvu II (1775 bp) fragment from the plasmid

25   pAGO containing all of the coding and polyadenylation
signals of the tk gene (with 57 nucleotides of 5'
untranslated region) is isolated and cloned together with
the XhoI -HpaII (2. 3Kb) fragment of the 6-16 promoter,
which spans all of the promoter region up to nucleotide -

30   4. The cloning is done in three steps. First, the BglII
to PvuII fragment of the HSV tk is subcloned into the
BamH1-Sma1 site of pUC18 to produce a plasmid named TK.pr⁻
. A diagnostic restriction carried with SmaI and EcoRI
should yield two fragments, one of 360 and another one of

35   4 Kb.

   Then, the fragment Xhol (preceded by the polylinker
sites HindIII to Sal1 of pUC18) to HpaII of the 6-16

promoter is subcloned into the AccI to HindIII sites of pUC18 to produce plasmid 6-16-Xho1-HpaII. Finally, the promoter of 6-16 is cloned in front of the tk gene by isolation of the Hind III-XbaI (2.3 kb) fragment from the plasmid 6-16.XhoI-HpaII into the XbaI-HindIII site of TK.pr⁻ to yield plasmid 6-16 TK, which contains the tk gene under the 6-16 promoter control.

        b.   Construction of a diphtheria toxin α gene
              driven by the 6-16 promoter

A similar approach to the cloning of the 6-16.TK is used to produce a DT-A gene driven by the 6-16 promoter. First, the 795 bp Bgl II fragment (flanked by EcoR1 and Hind III sites) is subcloned from the plasmid pDT-A (kindly provided by Dr. I Maxwell, University of Colorado). pDT-A contains the full coding sequence of the diphtheria α toxin gene. Because the toxin polypeptide is a polyprotein having both the α (24 kilodaltons) and β (38 kilodaltons) chains (Maxwell, 1987, Leong, 1983), the DNA is modified as follows to express the α chain only.

The initiator methionine codon is substituted from GTG to ATG, and the first two amino acids are changed to Asp-Pro. In addition, the DNA fragment contains at the carboxy terminal end a synthetic Ser-Leu -stop codon from small SV40 t antigen with a splice signal but devoid of polyadenylation signal (Maxwell, 1986, Palmiter, 1987).

To clone the DT-A insert, the DT-A is digested with HindIII, and then ligated to a 940 bp HindIII-EcoR1 SV40 fragment containing the polyadenylation sequences of T antigen, the remainder of the insert is recut with EcoR1. Consequently, the insert contains EcoR1 sites at the ends, and the DT-A fragment has a polyadenylation signal 3' of it. This EcoR1 1.7 kb fragment is cloned into the EcoR1 site of the 6-16 promoter plasmid.

The orientation of the insert is assessed by restriction with BglII. There are three BglII sites, one

at -603 in the 6-16 promoter and two at each end of the
DT-A 795 bp fragment. When the insert is in the right
orientation, BglII digestion should release the 600
fragment of the 6-16 promoter till the BglII of the DT-A

5   insert and the 795 by DT-A insert and the rest of the
plasmid. In the opposite orientation, the fragments
released will be of 795 (DT-A fragment) and 1.5 kb
(containing the 940 bp SV40 3' and polyadenylation
sequences + the 603 bp of the 6-16 promoter and the rest

10   of the plasmid.

    4.   Introduction of the Selected Genes Into the
          Target Cell

          The selected immunopotentiating and lethal genes may

15   be introduced into the cell by any of the methods known to
those of skill in the art. However, in a preferred
embodiment, cells will be transfected by the calcium
phosphate coprecipitation method (Graham, 1973),
lipofection, or electroporation. Tumor cells containing

20   selected transfected immunopotentiating genes, e.g. HA or
cytokines, may then be transfected with the lethal genes,
or, conversely, cells can first be transfected with the
lethal gene construct. Preferably, in addition to the
lethal gene, the lethal gene construct will contain

25   suitable marker and selection genes, which allow detection
and selection of transfectants. Preferred among these are
the marker plasmid pHC110 (Hall, 1983), containing the
bacterial β-galactosidase gene, and the selectable marker
pSV2hygro (Blochlinger, 1984). Cells are transfected with

30   those markers at a molar ratio of 15:15:1 and selected in
$400\mu g/ml$ hygromycin B. In addition, selected clones are
sensitive to gancyclovir or IFN and stain positively with
X-gal (Price, 1987, Thompson, 1989).

5.   Preclinical Studies

Because the molecular mechanisms of toxicity of the
diphtheria toxin and gancyclovir are different, a number
of different assays may be performed in order to determine
5   the concentrations of gancyclovir or IFN needed to obtain
the cytotoxic response in different cell types.   The
biological assays described below will be performed.

              a.   Assessment of concentration of Gancyclovir
                    necessary for killing cells transfected
10   with the Wild Type tk

The concentration of gancyclovir known to affect (by
more than 90%) the plating efficiency of mouse embryonal-
stem cells containing one copy of the HSV tk gene, is in
15   the range of 10^{-6} to 5×10^{-6} M.   The concentration of
gancyclovir needed to kill more than 90% of HSV-tk
transfected tumor cells will be determined by plating
efficiency assays and assays of $[^3H]$-thymidine
incorporation.   The plating efficiency assay is conducted
20   by plating 10^4 cells in 10 cm^2 plates, with increasing
concentrations of gancyclovir.   Every three days the media
is changed, and fresh gancyclovir added.   After 9 days,
the cells are stained with Giemsa, and the number of
colonies per plate is determined.   The assay of $[^3H]$-
25   thymidine incorporation is performed similarly to the
assay described below for biological fluids but using the
HSV-tk tumor transfected cells. .

              b.   Assessment of concentration of Gancyclovir
                    and IFN necessary for killing cells
30   transfected with the 6-16 tk ablation
                    vector

The incorporatic f GANC into DNA will probably
depend on both its extracellular concentration and the
35   intracellular concentration of the HSV tk enzyme.   Cells
having increased production of tk induced by addition of
IFN may have enhanced sensitivity to GANC.   This synergy
is studied using increasing concentrations of both

cytotoxic agents in conditions in which the concentration
of one of them remains constant. Increased toxicity will
be assessed both by [³H]-thymidine incorporation and
plating efficiency assays.

5

        c.   Determination of gancyclovir concentrations
             in biological fluids

     In order to be able to rapidly assess the
concentration of gancyclovir in biological samples such as
10   serum, [³H]-thymidine incorporation assays are set up with
LTK⁻ cells transfected with the HSV tk gene and selected
in HAT media. The assay is carried out in the presence of
aminopterin (1.8 ng/ml) and hypoxanthine (1.36 ng/ml) in
order to avoid the synthesis of dTMP from dUMP by
15   thymidine synthetase but to allow the synthesis of AMP and
GMP through the HGPRT purine salvage pathway. Under these
conditions the incorporation of [³H]-thymidine will depend
mainly on the amount of inhibition of DNA synthesis
produced by gancyclovir after its phosphorylation and
20   processing to a triphosphate derivative that inhibits DNA
polymerase, and the incorporation of [³H]-thymidine will
be independent of the internal UMP/TMP pools. The fact
that these cells do not contain an endogenous mammalian tk
gene will be of great advantage because the cells will be
25   extremely sensitive to gancyclovir.

     The assay for DNA synthesis is performed as follows.
Cells are seeded at $15\text{-}25 \times 10^3$ cells per well in a 96-
well microplate. The cells are incubated in triplicate
with or without known concentrations of gancyclovir in the
30   presence of hypoxanthine and aminopterin. After 24-28
hrs, cells are labeled with [3H]-thymidine 915 mCi/ml; 25
Ci/mmol; 1 Ci + 37 GBq; Amersham) for 1 hr. The cells are
then washed twice with phosphate-buffered saline, treated
with 5% trichloracetic acid for 30 min at 4°C, and washed
35   three times with 5% trichloroacetic acid. The precipitate
is dissolved in 0.1 ml of 0.2 M NaOH at 37°C for 30 min

and neutralized by 0.01 ml of 2 M CH1; radioactivity is
determined in a β-scintillation counter. The
concentration of gancyclovir from animal fluids could be
found by two fold dilutions of the samples and comparison

5   of the extent of inhibition obtained to the inhibition
curve of the standard used.

                d.   Assessment of concentration of IFN
                        necessary for inhibition of protein
10   synthesis and killing of cells transfected
                        with the 6-16 DT-A plasmid

        To investigate the dose of IFN and time of exposure
necessary to produce a cytotoxicity of more than 90%,
inhibition of protein synthesis in cells transfected with

15   the plasmid 6-16 DT-A is measured using a sensitive
colorimetric assay.
        Tumor cells cotransfected with the 6-16 DT-A plasmid,
pSV2neo (for selection in G418) and with pCH110, which
contains the bacterial β-galactosidase enzyme under the

20   control of the early SV40 promoter, are selected by
culture in G418. Resistant cell clones are isolated and
tested by southern blotting to prove that all plasmids are
present. Selected cell clones are those which are killed
by IFN and have detectable levels of β-galactosidase

25   activity.
        The inhibition of protein synthesis due to the
activation of the diphtheria toxin will produce a decrease
in the amount of βgalactosidase that can be detected in a
colorimetric assay using the substrate pNPG (p-

30   nitrophenyl-β-D-galactopyranoside). This substrate of β-
galactosidase turns yellow upon cleavage by the enzyme and
the accumulation of the yellow product can be measured at
a wavelength of 220 nm in Elisa plates. This enzymatic
system will also be useful in vivo. The presence of cells

35   containing the β-galactosidase gene can be visualized in
histological samples by using the substrate X-gal that,
upon cleavage, produces a blue precipitate in the cells.

Therefore, this assay allows the determination of the presence of tumor cells containing lethal gene constructs in situ in animal tissue after treatment with IFN.

5   6.   In vivo experiments
           The following prophetic examples describe practice of the invention in a variety of murine tumor models.
           CT-26 cells with or without immunopotentiating and/or lethal genes are injected into syngeneic BALB/c animals.

10   Similarly, SP1 and RENCA cells described below are injected into their syngeneic hosts CBA and BALB/c mice, respectively. To estimate the immunogenic potential of the cells expressing the lethal genes, 1×10^3 to 10^7 cells will be injected in the right flank of the animals while

15   the parental nontransfected cells will be injected on the left flank. The number of tumor cells required to kill in 50% of the animals will be estimated by the method of Reed and Muench (Reed, 1938) as previously described (Fearon, 1988).

20

           7.   Models
                a.   H4A
           The H4A cell line was derived by transfection of the SP1 mammary adenocarcinoma with the influenza virus

25   hemagglutinin (HA) gene. Cells expressing HA were selected four times using Fluorescence Activated Cell Sorting (FACS) as previously described (Fearon, et al., 1989), and the H4A cell line was obtained. H4A fails to grow in syngeneic hosts at a challenge dose of 1×10^4,

30   but does grow if 1×10^5 H4A cells are injected subcutaneously. Despite the growth of the cells, the animals do mount a cytotoxic T cell response, though this response is inadequate or occurs too late to offset tumor growth.

35   CBA mice will be injected with H4A, or H4A transfected with either HSV-tk or DTA. When the resultant

tumor reaches different sizes (2-4 mm, 6-8 mm, 1 mm or 2 mm) all animals will be treated in the following manner to kill the injected cells.

TUMOR CELL INJECTED	TREATMENT
H4A	None
H4A	GANC
H4A	IFN-α
H4A	GANC + IFN-α
H4A - HSV tk	None
H4A - HSV tk	GANC
H4A - HSV tk	IFN-α
H4A - HSV tk	GANC + IFN-α
H4A-DTA	None
H4A-DTA	GANC
H4A-DTA	IFN-a
H4A-DTA	GANC + IFN-a

The initial doses of IFN-a (Hoffman-LaRoche, Nutley N.J.) will be 5×10^4 units/injection s.c. three times a week for two consecutive weeks. GANC (Syntex, Palo Alto, CA) will be given s.c. at 20 mg/kg/day initially.

Animals in whom the primary tumor has been rejected are then:

a) be assessed for a splenic cytotoxic T cell response;

b) be challenged with 1×10^4, 1×10^5, or 5×10^5 parent cells to detect immunity to a parent tumor challenge.

b. CT-26/IFN-γ

The CT-26 murine colon carcinoma, when transfected with and expressing IFN-γ, becomes partially immunogenic. While a 1×10^5 challenge dose of parental CT-26 cells is lethal to normal animals in 3 weeks; 60% of the CT-26 IFN-γ cell injected animals survive greater than 8 weeks. These cells appear to be immunogenic, but the response

they engender is sometimes insufficient to prevent tumor
growth. In addition, animals immunized with viable CT-
26/IFN-γ were not protected against a parent CT-26
challenge. Furthermore, irradiated CT-26/IFN-γ cells were
also nonprotective against a challenge with parental
cells.

        c.   RENCA Model
        RENCA is a renal cell carcinoma of BALB/c mice. The
injection of 5 x 10⁴ RENCA cells into the subrenal capsule
of syngeneic mice results in the development of a u 1 cm
tumor with two weeks. If the tumor bearing kidney is
surgically removed, the animals will all develop extensive
lung metastases within two weeks.

C.   Human Immunotherapy
        Due to the necessary constraints imposed on human
therapy, the present invention has not yet been tested in
human subjects. However, it is contemplated that the
invention may be used in humans having selected tumors,
for example, of the colon, breast, ovary, melanoma, and
sarcoma. Generally, it is preferred to select for
treatment patients who have had a discrete primary tumor
removed for which there is a high risk of recurrence. In
a usual situation, tumor cells from the patient will be
isolated and transfected with suitable immunopotentiating
and lethal genes. Of course, it will be appreciated that
when the immunopotentiating gene is a cytokine, such as
interleukin-2, a gene encoding a human cytokine will be
used. After transfection, the cells will be selected to
obtain cell lines which are stably transfected with the
selected genes.
        In the ideal situation, the patient will first be
treated with any of a number of regimes designed to reduce
tumor burden, such as surgical resection, irradiation,
etc., prior to administration of the tumor cell immunogen.

The cells are then allowed to immunize the host for a
selected period of time, usually about 14 days.
Throughout this period of time, suitable assays may be
performed to assess the immune status of the patient to
5   the particular tumor in question. However, it should be
realized that where the immunization is local in nature,
systemic immunity may not be observed in all cases, and it
may thus be necessary to obtain lymphocytes from the tumor
site for such studies. In any event, after a selected
10   period of time in which the host has been effectively
immunized against its tumor cells, the lethal gene will be
caused to eliminate the remaining immunizing tumor cells.
In the instance when the selectably inducible promoter
employed is a 6-16 promoter described here, alpha
15   interferon will be administered to the patient in a dose
effective to initiate transcription of the lethal gene but
not so high as to be acutely toxic to the patient. The
alpha interferon may also be administered locally to the
site of the vaccine introduction. When the Herpes simplex
20   thymidine kinase gene is used, it will be necessary to
administer gancyclovir. When the thymidine kinase gene is
driven by the 6-16 promoter, administration of both
gancyclovir and interferon alpha will be warranted.
While definite parameters for each of these
25   administrations and the like have not yet been
ascertained, they should be readily ascertainable by those
of ordinary skill in the art with the assistance provided
here.

30   * * * * * * * * *

The foregoing description of the invention has been
directed to particular preferred embodiments in accordance
with the requirements of the patent statutes and for
purposes of explanation and illustration. It will be
35   apparent, however, to those skilled in the art that many

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modifications and changes may be made without departing
from the scope and the spirit of the invention.

    For example, numerous methods for introducing the
exogenous genes into the target cells may be used. In
5   addition, various selected genes may be transfected in
accordance with the invention. For example, certain
aspects of the invention, such as the provision of a
selectively inducible lethal gene, may not be limited to
use in immunotherapy but may be more widely applicable to
10 any situation in which it is desirable to be able to
specifically ablate a genetically engineered cell in vivo.
In addition, the transfected cell lines of the present
invention are useful for ex vivo immunization, for
example, in activation of LAK cells for infusion into a
15 patient according to the procedure described in U.S.
Patent # 4,690,915, issued to Rosenberg, or in vitro
activation of cytotoxic T lymphocytes or for promoting
growth of cytokine dependent cell lines, such as IL-2
dependent cell lines. The ability to purge in vitro
20 cultures of the transfected cells by simply inducing
transcription and translation of the lethal gene provides
an additional advantage. Therefore, it is apparent that
the invention may also be utilized, with any number of
suitable modifications within the state of the art. It is
25 the Applicants intention in the following claims to cover
all such equivalent modifications and variations which
fall within the true spirit, and scope of the invention.

    REFERENCES

30   The following references may facilitate understanding
or practice of certain aspects of the present invention.
Inclusion of a reference in this list is not intended to
and does not constitute an admission that such reference
represents prior art with respect to the present
35 invention.

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5

CLAIMS:

1.   A composition for potentiating the immune response to a tumor, the composition comprising a cell derived from the tumor, the cell including a first exogenous gene encoding an immunopotentiating polypeptide and a second exogenous gene including a promoter in operative linkage to DNA encoding a polypeptide capable of killing the cell upon induction of the promoter.

2.   The composition of claim 1 wherein the promoter is a selectively inducible promoter.

3.   The composition of claim 2 wherein the inducible promoter is a 6-16 interferon inducible promoter.

4.   The composition of claim 2 or claim 3 wherein the second exogenous gene includes a selectively inducible promoter in operative linkage to a gene encoding the alpha chain of diphtheria toxin.

5.   The composition of claim 2 or claim 3 wherein the second exogenous gene includes a selectively inducible promoter in operative linkage to a gene encoding Herpes simplex virus thymidine kinase.

6.   The composition of claim 1, claim 2, or claim 3, wherein the immunopotentiating polypeptide is a cytokine.

7.   The composition of claim 6 wherein the immunopotentiating polypeptide is an interleukin.

8.   The composition of claim 7 wherein the interleukin is interleukin-1.

9.   The composition of claim 7 wherein the interleukin is interleukin-2.

10.   The composition of claim 7 wherein the interleukin is interleukin-3.

11.   The composition of claim 7 wherein the interleukin is interleukin-4.

12.   The composition of claim 7 wherein the interleukin is interleukin-5.

13.   The composition of claim 7 wherein the interleukin is interleukin-6.

14.   The composition of claim 1, 2, or 3 wherein the immunopotentiating polypeptide is an antigen alien to the host of the tumor.

15.   The composition of claim 14 wherein the antigen is a histocompatibility antigen.

16.   The composition of claim 14 wherein the antigen is a viral envelope protein antigen.

17.   The composition of claim 14 wherein the antigen is a bacterial antigen.

18.   The composition of claim 17 wherein the antigen is the 65kDa antigen of Mycobacterium tuberculosis.

19.   The composition of claim 1 wherein the genome includes a third exogenous gene, encoding a second immunopotentiating polypeptide.

20.   The composition of claim 1 wherein the second exogenous gene encodes an immunopotentiating antigen if the first exogenous gene encodes an immunopotentiating cytokine and wherein the second exogenous gene encodes an

immunopotentiating cytokine if the first exogenous gene encodes an immunopotentiating antigen.

21. A cell from a vertebrate organism comprising a first exogenous gene encoding a selected polypeptide and a second exogenous gene comprising a promoter in operative linkage to DNA encoding a second selected polypeptide, the second selected polypeptide capable of killing the cell.

22. The cell of claim 21 wherein the promoter is a selectively inducible promoter and the second polypeptide is capable of killing the cell upon induction of the promoter.

23. The cell of claim 22 wherein the promoter is 6-16 interferon promoter.

24. The cell of claim 21 wherein the second polypeptide is capable of killing the cell upon addition of a substrate for the polypeptide.

25. A method for producing a cell for gene therapy of a selected disease comprising:
   a.   introducing a first exogenous gene encoding a selected polypeptide into a cell; and
   b.   introducing a second exogenous gene containing a promoter in operative linkage with DNA encoding a polypeptide capable of killing the cell into the cell to produce a cell having both the first and the second exogenous genes maintained in the cell.

26. The method of claim 25 wherein said promoter is a selectively inducible promoter.

27. The method of claim 26 comprising the additional step of inducing the promoter so as to kill the cells.

28. A method for gene therapy of a vertebrate organism comprising administering the composition of any one of claims 21 -27 to the organism.

29. A method for gene therapy of a vertebrate organism comprising:

      a.   introducing a first exogenous gene encoding a selected polypeptide into a cell; and

      b.   introducing a second exogenous gene containing a promotor in operative linkage with DNA encoding a polypeptide capable of killing the cell into the cell to produce a cell having both the first and the second exogenous genes stably maintained in the cell; and

      c.   administering the cell to the organism.

30. The method of claim 29 wherein the promoter is a selectively inducible promoter.

31. The method of claim 30 comprising the additional step of inducing the promoter.

32. A cellular preparation for potentiating an immune response of a vertebrate organism to a tumor comprising a preparation of cells, derived from the tumor, containing an exogenous gene encoding an immunopotentiating interleukin.

33. A cellular preparation for potentiating the immune response of a vertebrate organism to a tumor comprising a preparation of cells, derived from the tumor, containing an exogenous gene encoding interleukin-2.

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34.   The composition of claim 32 or claim 33, wherein the exogenous gene is introduced into the cells by transfection.

35.   A composition for enhancing non-specific resistance of a vertebrate organism to growth of a tumor comprising a preparation of cells including an exogenous gene encoding interferon.

36.   The composition of claim 35 wherein the interferon is interferon γ.

37.   The composition of claim 35 wherein the exogenous gene is introduced into the tumor by transfection.

38.   The composition of claim 32 or claim 35 wherein the cells further comprise an exogenous gene encoding an additional immunopotentiating polypeptide.

39.   The composition of claim 38 wherein the additional immunopotentiating polypeptide is an antigen foreign to the mammal.

40.   The composition of claim 38 wherein the antigen is a viral envelope protein.

41.   The composition of claim 38 wherein the antigen is a bacterial cell antigen.

42.   The composition of claim 38 wherein the antigen is the 65kDa antigen of Mycobacterium tuberculosis.

43.   The composition of claim 38 wherein the immunopotentiating polypeptide is a cytokine.

44.   The composition of claim 38 wherein the cytokine is
interleukin-1.

45.   The composition of claim 38 wherein the cytokine is
interleukin-3.

46.   The composition of claim 38 wherein the cytokine is
interleukin-4.

47.   The composition of claim 38 wherein the cytokine is
interleukin-5.

48.   The composition of claim 38 wherein the cytokine is
interleukin-6.

49.   The composition of claim 38 wherein the cytokine is
interferon.

50.   The composition of claim 38 wherein the cytokine is
gamma interferon.

51.   A method for potentiating the immune response of a
vertebrate organism to a tumor comprising the steps of
introducing into the organism a viable preparation of the
composition of any one of claims 32-37.

52.   A method for potentiating the immune response of a
vertebrate organism to a tumor comprising the steps of
introducing into the organism a viable preparation of the
composition of claim 38.

53.   The method of claim 51 comprising the additional
steps of reducing the number of tumor cells in the
vertebrate organism prior to introducing the preparation.

54.   The method of claim 53 wherein the reduction
comprises surgical resection.

Table 1.   Growth of CT26, CT26-IL-2$^+$, and CT26-neo-IL-2$^-$ in BALB/c Mice

Tumor	Injection Dose[a]	# Mice with Tumor/ # Mice Injected
CT26	1×10^3 cells	9/10[b]
	1×10^4 cells	20/20[c]
	1×10^5 cells	25/25[c]
	1×10^6 cells	20/20[c]
CT26-IL-2$^+$	1×10^4 cells	0/20[e]
	1×10^5 cells	0/35[e]
	1×10^6 cells	2/55[e]
	1×10^7 cells	4/15[e]
CT26-neo-IL-2$^-$	1×10^4 cells	10/10[b]
	1×10^5 cells	20/20[d]
	1×10^6 cells	10/10[d]

[a]   BALB/c mice were injected subcutaneously on the left hind leg, and tumor growth was observed.

[b]   All mice had tumors by 3 weeks.

[c]   All mice had tumors by 2 weeks.

[d]   Most mice had tumors by 2 weeks, all had tumors by 3 weeks.

[e]   Tumor-free mice were observed from 6-12 weeks.

Table 2. In Vivo Growth, Protective Immunity, and CTLs
Generated by IL-2-Transfected B16 Melanoma Cells

	# Cells Injected[b]	Tumor Growth[c]	Protection against Challenge with B16[d]	Lysis of B16[e]
B16	1×10^4	4/5	---	---
	1×10^5	5/5	---	16%
	1×10^6	5/5	---	---
B16-IL-2[+a]	1×10^4	0/9	9/9	---
	1×10^5	0/8	6/8	26%
	1×10^6	0/9	5/9	---

[a] B16-IL-2^{+} transfectant was produced by transfection
with pBCMG-neo-mIL-2 as described in Experimental
Procedures.

[b] Cells were injected subcutaneously into the left flank
as in Table 1.

[c] Tumor growth was measured weekly. Animals were
observed for 7 weeks. All animals that developed tumors
did so by 2 weeks after injection.

[d] Challenge with the 1×10^5 parental B16 cells was done
in the right flank 2 weeks after immunization with B16-IL-
2^{+} cells as in Figure 3. Animals were observed for 7
weeks after challenge. Numbers represent animals that
acquired tumors. These tumors were observed to arise
between 3 and 5 weeks after challenge. Tumor-free animals
were observed for 7 weeks.

[e] CTL assays were performed after a 5 day stimulation
with mitomycin C-treated B16 cells as in Figure 1a.
Numbers represent % specific lysis after an 8 hr ^{51}Cr
release assay at 100:1 effector to target ratio.

TABLE 3

Growth of IFN_γ Transfected SP1 Tumor Cells

Tumor	IFN_γ Production (Units/ml)	No. of mice with tumor/ No. of mice challenged		
		10^5	5×10^5	10^6
SP1 Parent	---	10/10	NT	NT
$SP1/IFN_\gamma$				
1C	16	2/3*	NT	NT
3C	32	3/3*	NT	NT
6L	256	0/15	0/10	1/15°

Groups of CBA mice were injected s.c. with either 10^5, 5×10^5 or 10^6 cells from three different transfection plates (1C, 3C and 6L). The animals were then observed for tumor growth for 90 days.

* Growth at 24 days

° Growth at 90 days

TABLE 4

Induction of Cytotoxic T Cells by IFN$_\gamma$ producing SP1 cells

Tumor cells used for immunization	Target Cells	Effector: Target Ratio	% Killing		
			Whole Spleen Cells	NWNA	NWA
CBA	CBA	100:1	2.3	---	---
6L	CBA	100:1	30	---	---
		50:1	19	19	64
		10:1	12	8	39
6L	6L	100:1	40	---	---
		50:1	16	24	60
		10:1	0	13	38
6L	MDW1	50:1	2.2	2.4	19
		10:1	1.8	2.7	8

Groups of CBA mice were injected s.c. with either 5 x 10^5
SP1 or SP1/IFN$_\gamma$ (6L) cells. Two weeks later the animals
were killed and their spleen cells incubated with
irradiated SP1 or 6L cells. Five days later the
lymphocytes were harvested and passed through a nylon wool
column and adherent and nonadherent spleen cells were
added to the ^{111} Inox labeled targets listed, at the ratios
indicated.

   NWA - Nylon Wool Adherent

   NWNA - Nylon Wool Non-Adherent

TABLE 5

Immunoprotection by IFN$_\gamma$ transfected SP1 cells against a challenge with parent cells

Tumor	Immunization dose		Challenge Dose		
	1st	2nd	10^4	5×10^4	10^5
SP1	---	---	10/10*	10/10	10/10
SP1/IFN$_\gamma$ 6L	2×10^6	10^6	9/18	10/10	18/19

CBA mice were immunized with 10^6 6L cells on day 0 and again on day 14. On day 19, immunized and control animals were challenged with 10^4 or 10^5 parent SP1 cells. The animals were then observed for tumor growth.

* Number of mice with tumors/Number of mice challenged.

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TABLE 6

Growth of IFN_γ producing CT-26 tumor cells

Tumor cells	Challenge dose	Route	Tumorigenicity		
			3w	5w	7w
CT-26	1×10^5	s.c.	9/10*	---	---
CT-26/IFN_γ	1×10^5	s.c.	2/15	---	9/15
CT-26	5×10^4	i.v.	---	5/5	---
CT-26/IFN_γ	5×10^4	i.v.	---	0/5	3/5

Groups of BALB/c mice were injected s.c. or i.v. with CT-26 or CT-26IFN_γ cells and observed for tumor growth and survival.

*   Number of mice with tumors/Number of mice challenged.

FIG.1A

FIG.1B

2 / 7

FIG.1C

FIG.2

2 WEEKS POST IMMUNIZATION

4 WEEKS POST IMMUNIZATION

NO PREIMMUNIZATION

WEEKS POST RIGHT FLANK
CHALLENGE WITH CT26

FIG.3

CT26-IL2+/ANTI CD4+8   CT26-HA3/ NO Ab

CT26-IL2+/NO Ab

CT26 -IL2+/ANTI CD4

CT26-IL2+/ANTI CD8

CT26-HA3/
ANTI CD4

PERCENT WITH TUMOR < 0.5 cm

WEEKS POST LEFT FLANK INJECTION

4 / 7

FIG.4A

SP1 PARENT

RELATIVE CELL NUMBER

FLUORESCENCE INTENSITY (log)

FIG.4B

SP1 +IFN-γ

RELATIVE CELL NUMBER

FLUORESCENCE INTENSITY (log)

FIG.4C

CT-26 PARENT

RELATIVE CELL NUMBER

FLUORESCENCE INTENSITY (log)

FIG.4D

CT-26/IFN-γ

RELATIVE CELL NUMBER

FLUORESCENCE INTENSITY (log)

5/7

FIG. 5A

FIG. 5B

FIG. 5C

FIG. 5D

FIG. 5E

FIG. 5F

SUBSTITUTE SHEET

FIG.6A

RELATIVE CELL NUMBER

SP1 PARENT

FLUORESCENCE INTENSITY (log)

FIG.6B

RELATIVE CELL NUMBER

SP1/IFN-γ 3C

FLUORESCENCE INTENSITY (log)

FIG.6C

RELATIVE CELL NUMBER

SP1/IFN-γ 1C

FLUORESCENCE INTENSITY (log)

FIG.6D

RELATIVE CELL NUMBER

SP1/IFN-γ 6L

FLUORESCENCE INTENSITY (log)

7 / 7

FIG.7A

IFN – γ
4u/ml
6L-A

FLUORESCENCE INTENSITY (log)
(GROWTH IN CBA MICE +)

RELATIVE CELL NUMBER

FIG.7B

IFN – γ
256u/ml
6L-B

FLUORESCENCE INTENSITY (log)
(GROWTH IN CBA MICE –)

RELATIVE CELL NUMBER

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/06612

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5   C 12 N 15/85   A 61 K 48/00   A 61 K 35/12
C 12 N 5/10 // C 12 N 15/19   C 12 N 15/31   C 12 N 15/54

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols		
Int.Cl.5	A 61 K	C 07 K	C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Cell, volume 60, 9 February 1990, Cell Press (Cambridge, NA, US) E.R. Fearon et al.: "Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response", pages 397-403, see the whole article (cited in the application)	32-34
Y		1,2,4,6 ,7,9,29

X	Proceedings of the National Academy of Sciences, volume 86, December 1989 (washington, US) Y. Watanabe et al.: "Exogenous expression of mouse interferon gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity", pages 9456-9460, see the whole article	35-37
	--- -/-	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot we considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24-01-1992	12. 02. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Maria Pels *Maria Pez*

III. DOCUMENTS CONSIDERED TO BE RELEVANT	(CONTINUED FROM THE SECOND SHEET)	
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Immunology Today, volume 11, no. 6, June 1990, Elsevier Science Publishers Ltd (GB) S.J. Russell: "Lymphokine gene therapy for cancer", pages 196-200, see especially pages 196-197 ---	32-37
X	Cancer Research, volume 46, no. 9, September 1986, (Philadelphia, US) I.H. Maxwell et al.: "Regulated expression of a diphtheria toxin A-chain gene transfected into human cells: possible strategy for inducing cancer cell suicide", pages 4660-4664, see the abstract; page 4661, lines 10-13	21,22, 24-27
Y		1-2,4, 14-16,6 -7,9,29
A	--- Cancer Research, volume 48, no. 11, 1 June 1988 (Philadelphia, US) E.R. Fearon et al.: "Induction in a murine tumor of immunogenic tumor variants by transfection with a foreign gene", pages 2975-2980, see the whole article, especially page 2979, lines 43-47; page 2980, lines 23-30 (cited in the application)	38-40
Y		1-2,14-16
A	--- Chemical Abstracts, volume 112, no. 19, 7 May 1990 (Columbus, Ohio, US) F.L. Moolten et al.: "Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors", see page 36, abstract 171876a, & J. Natl. Cancer Inst. 1990, 82(4), 297-300 ---	5
A	Biological Abstracts, volume 90, no. 5, 1990, AB-637; S. Ostrand-Rosenberg et al.: "Transfection of major histocompatibiity complex class I and class II genes causes tumor rejection", see left-hand column, abstract no. 52893, & J. Immunogenet. 16(4/5) 1989, pages 343-350 ------	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers   because they relate to subject matter not required to be searched by this
Authority, namely:

    Although claims 28-31, 51-54 are directed to a method of
    treatment on the animal body the search has been carried
    out on the alleged effects of the composition.

2. ☐ Claim numbers   because they relate to parts of the International application that do not comply
with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers   because they are dependent claims and are not drafted in accordance with
the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims
of the International application

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only
those claims of the International application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to
the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not
invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) - P9412B 05/81